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EDITORIAL REVIEW

Proteolytic enzymes as mediators of glomerular injury

Despite significant advances in our understanding of the pathogenesis of glomerular injury, the specific mechanisms responsible for the pathological changes in glomerular structure and function which occur in glomerular disease remain poorly understood. Several diverse lines of evidence, including studies with purified proteinases, studies with freshly isolated glomeruli and intact cells, and cells in culture, as well as recent in vivo studies indicate that proteolytic enzymes play important roles as mediators of glomerular injury. The concept of proteolytic enzymes as mediators of glomerular injury is not new. The ability of polymorphonuclear leukocyte (PMN)-derived proteinases to degrade glomerular basement membrane (GBM) in vitro coupled with the PMN-dependence of certain types of glomerulonephritis led Cochrane and coworkers in the mid-sixties to postulate that neutrophil-derived proteinases cause glomerular injury by proteolysis of the GBM [1–3]. Since then, a large number of studies have provided support for this hypothesis. In addition, recent studies documenting the presence of GBM-degrading proteinases in glomeruli, plasma, and a variety of other circulating cells indicate that GBM damage can also be mediated by proteinases originating from these sources as well. Thus proteinase-mediated GBM damage may be an important mechanism of glomerular injury in leukocyte-independent as well as leukocyte-dependent glomerular diseases. Furthermore, recent studies suggest that proteolytic enzymes may contribute to glomerular injury by mechanisms independent of GBM damage. Such mechanisms include: proteolysis of non-GBM proteins; proteinase-mediated alterations in the production of key glomerular metabolites; and proteinase-induced effects on cellular proliferation. In the present review, we attempt to bring together a large body of diverse information derived from in vitro and in vivo studies, which supports a pathogenic role for proteolytic enzymes in glomerular injury.

Proteolytic enzymes: Nomenclature and classification

Proteolytic enzymes, (peptidases) are classified as exo- or endopeptidases. Exopeptidases are proteinases which cleave peptide bonds close to the end of a polypeptide chain. Endopeptidases (also referred to as proteinases and proteases) cleave internal peptide bonds. Although many exopeptidases are present in the kidney and may play important roles in glomerular injury, most studies concerning the role of proteolytic enzymes in glomerular injury have focused on endopeptidases. In contrast to most enzymes, which are named and classified according to their substrate and the type of reaction catalyzed (such as alcohol dehydrogenase), endopeptidases are classified according to their catalytic mechanisms and named for the essential catalytic component (usually an amino acid) in their active site [4]. Table 1 lists the four classes of endopeptidases and some general properties of each class.

Potential sources of proteolytic enzymes for glomerular injury

With few exceptions, a proteolytic enzyme must have assess to the glomerulus in order to produce glomerular injury. As mentioned above, the PMN has long been recognized as a source of proteolytic enzymes available for glomerular injury [1–3, 5]. However, other circulating blood cells such as monocytes, macrophages, and platelets contain a variety of proteolytic enzymes [6] and thus must also be included as potential sources of proteolytic enzymes. In addition, recent studies from our laboratory have demonstrated that glomeruli contain several proteolytic enzymes, most notably metalloproteinases [7, 8] and cysteine proteinases [9], which may also contribute to glomerular injury. Thus the glomerulus itself, including mesangial, endothelial, and epithelial cells constitutes a second important source of proteolytic enzymes available for glomerular injury. A third potential source of proteolytic enzymes is the plasma. Well-documented proteolytic enzymes in plasma include components of the coagulation, complement, and fibrinolytic systems. Although these plasma proteinases circulate as zymogens (inactive forms) and are usually activated only as part of well-regulated physiological processes, several lines of evidence suggest that they participate in processes which may contribute to glomerular injury. For example, thrombin appears to play an important role in the intraglomerular fibrin deposition which occurs in certain types of glomerulonephritis [10]. In addition, recent studies suggest that plasmin, generated from plasma plasminogen by glomerular plasminogen activators, may also play an important role(s) in glomerular injury. Table 2 includes a list of several proteinases of potential interest to glomerular injury as well as potential sources of each of these proteinases.

It is important to recognize that interactions between proteolytic enzymes, including those from different sources, can and do occur. For example, Gavrilovic et al [11–13] have demonstrated that the degradation of type I collagen films by phorbol myristate acetate- or interleukin-1-stimulated cells in culture is dependent on the presence of plasminogen in the medium. However, collagen degradation was inhibited by the collagenase inhibitors TIMP (tissue inhibitor of metalloproteinases) and antibodies to collagenase. Based on these observations, these workers postulated that plasminogen activator(s), produced by the stimulated cells, converted plasminogen to plasmin which in turn activated procollagenase (also produced by the cells) which is directly responsible for the collagen degradation. Clearly the potential for such interactions (and others as discussed below) must be recognized in the design of experimental protocols as well as the interpretation of results.

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Table 1. Classes and some general properties of endopeptidases[a]

Property	Serine	Cysteine	Aspartic	Metallo
Old name	Serine	Thiol	Carboxyl	Metallo
Enzyme commission #	3.4.21	3.4.22	3.4.23	3.4.24
Active site component	Serine	Cysteine	Aspartic acid	Zn^{2+}
pH range[b]	7–9	3–7	2–6	5–9
Inhibitors (in vitro)	PMSF, SBTI, DIFP	E-64, Iodoacetate, organo-mercurials	Pepstatin	EDTA, o-phenanthroline
Inhibitors (in vivo)	Plasma proteinase inhibitors[c]	Cystatins, α_1-cysteine proteinase inhibitor	Unknown	TIMP-1, TIMP-2
Location	Intra- and extracellular	Lysosomes[d]	Lysosomes	Intra- and extracellular
Latent forms	Yes	Yes	No	Yes
Examples	Elastase, plasmin	Cathepsins B & L	Cathepsin D	Gelatinase, type IV collagenase

[a] Adopted from [4]
[b] Varies with substrate and assay conditions
[c] α_1-Protenase inhibitor, α_2-antiplasmin, α_2-macroglobulin, etc. [106]
[d] Except calpain, the cytosolic & membrane-bound Ca^{2+}-dependent cysteine proteinase [155]

Table 2. Proteolytic enzymes potentially involved as mediators of glomerular injury

			Proposed mechanism of glomerular injury[a]		
Proteinase	Class	Source	Proteolysis of GBM	Proteolysis of nonGBM proteins	Altered glomerular metabolism
Elastase & cathepsin G	Serine	Leukocytes	5, 33–40, 57, 58, 60, 61	41, 42, 85, 93, 109, 112, 114, 115	23, 25, 26, 125, 131
Plasmin	Serine	Plasma	94–99	10–13, 102–104	
Thrombin	Serine	Plasma	95	10	20–22, 24, 27, 29, 138
Gelatinase	Metallo	Leukocytes	41, 56, 59–61	113	
Gelatinase	Metallo	Glomeruli	50–52		
Glomerular metalloproteinase(s)	Metallo	Glomeruli	7, 8		
Cathepsins B & L	Cysteine	Glomeruli	9, 67–71	102, 111	

[a] Numbers refer to reference number

Potential mechanisms of proteinase-mediated glomerular injury

In vitro studies have delineated a variety of biological effects of proteolytic enzymes of potential relevance to glomerular injury. For purposes of discussion, we have grouped these effects into three general categories of potential mechanisms of proteinase-mediated glomerular injury: proteolysis of the GBM; proteolysis of non-GBM proteins; and proteinase-mediated alterations in glomerular metabolism, (including effects on cell proliferation). It is important to point out that these mechanisms are not mutually exclusive. As described below and listed in Table 2, the ability of several proteolytic enzymes (such as elastase, plasmin) to produce effects described in more than one category is well documented.

Proteolysis of the GBM

The GBM is the major filtration barrier of the glomerulus restricting the passage of plasma proteins into the urine [14–16]. The hallmark of glomerular disease is proteinuria resulting from an increase in the permeability of the GBM to plasma proteins. Thus, possibly the most obvious mechanism by which proteolytic enzymes can cause glomerular injury is degradation of the GBM. The GBM is composed primarily of type IV collagen, with lesser amounts of laminin, entactin (nidogen), and heparan sulfate proteoglycan. Fibronectin, collagen types V and VII, osteonectin (SPARC, BM-40) and several other proteins are also present in GBM, although it is presently unclear whether these proteins are intrinsic GBM components or passively accumulated following GBM synthesis [17–19]. It should be noted that each component of the GBM, including heparan sulfate proteoglycan, is susceptible in vitro to proteolysis by one or more proteinases available to or present in normal glomeruli. As discussed in detail below, a large body of evidence suggests that proteinase-mediated GBM damage is an important mechanism of glomerular injury.

Proteolysis of non-GBM proteins

In addition to GBM degradation, proteolysis of non-GBM proteins can also occur. For example, inactivation of proteinase inhibitors could result in glomerular injury mediated by uncontrolled proteinase activity. In vitro the ability of proteolytic enzymes to inactivate proteinase inhibitors is well documented (see below) and raises the possibility that such inactivation may represent an additional mechanism by which proteinases could contribute to glomerular injury.

In contrast to the proteolysis of GBM and proteinase inhibitors, which results in loss of function, proteolysis of many proteins results in increased activity of the target protein. Pertinent examples are the proteinase-mediated activation of

zymogens, the inactive forms of many enzymes. Normally zymogen activation occurs by limited proteolysis, carried out by a specific proteinase as part of a well controlled physiological process, such as coagulation or fibrinolysis. However, "inappropriate" activation of zymogens does occur and can lead to pathological changes, as evidenced by intraglomerular fibrin deposition resulting from the localized activation of thrombin [10].

Thus proteolysis of non-GBM proteins, resulting in either activation or inactivation of the target protein, represents a second potential mechanism of proteinase-mediated glomerular injury.

Proteinase-mediated alterations in glomerular metabolism

The ability of proteolytic enzymes to influence the production of several key metabolites, autocrine mediators, and intracellular signalling agents is well documented. For example, addition of proteolytic enzymes to intact cells in vitro, has resulted in altered production and/or secretion of cyclic nucleotides [20, 21], prostaglandins [22–24], platelet activating factor [25], Ca^{2+} flux and inositol polyphosphate synthesis [26, 27], adenine nucleotides [23], and reactive oxygen metabolites [28]. Each of these metabolites is postulated to play an important role in glomerular pathophysiology. In addition, recent studies [27, 29] indicate that certain proteolytic enzymes stimulate the proliferation of cultured glomerular cells. Although only limited data is available documenting altered metabolism in intact glomeruli or glomerular cells, similarities in the pathways for the production and catabolism of these metabolites among various tissues and the well-documented ability of proteinases to alter metabolism in other tissues suggests that this may be an important mechanism of proteinase-mediated glomerular injury.

In vitro evidence for proteinase-mediated glomerular injury

Proteolysis of GBM and GBM components

GBM degradation in leukocyte-dependent glomerulonephritis. Leukocytes contain proteinases in each of the four classes [6] which have been shown to effectively degrade GBM in vitro under appropriate conditions. However, several lines of evidence suggest that the serine proteinases elastase and cathepsin G and the metalloproteinase gelatinase are the leukocyte proteinases most likely to contribute to GBM damage in vivo.

Elastase and cathepsin G are well characterized serine proteinases [reviewed in 30–32] present in neutrophils. In addition, proteinases with properties similar to elastase and cathepsin G have been reported in several tissues and cells types, including macrophages, monocytes, and platelets [30–32]. Three properties of elastase and cathepsin G are of particular interest with respect to their ability to damage the GBM: their neutral pH optimum which facilitates their action in neutral pH environments such as the plasma-GBM interface; their positive charge at plasma pH (pIs = 9 to 11) which facilitates electrostatic binding to the negatively charged GBM; and their relatively low molecular weights (27 to 35 kDa) which promotes penetration into the GBM lattice and possibly allows them to escape from higher molecular weight plasma proteinase inhibitors. Thus the physical properties of these proteinases combined with their broad substrate specificity (see below) render them ideal candidates for mediators of glomerular injury by damage to the GBM.

GBM degradation at neutral pH by neutrophil granule extracts (now known to contain large amounts of elastase and cathepsin G) was first reported by Janoff and Zeligs [5]. This observation was confirmed and extended in subsequent studies by Davies, Coles and Hughes [33], who demonstrated that lysates of human PMN or monocytes as well as highly-purified human PMN elastase and cathepsin G could effectively degrade GBM at neutral pH in vitro. In addition, neutrophil granule extracts, purified elastase, and/or cathepsin G have been shown to degrade specific components of the GBM including proteoglycans [34–36], fibronectin [37, 38]; and type IV collagen [39, 40].

Neutrophils contain two other major neutral proteinases: the metalloproteinases, collagenase and a 92-95 kDa gelatinase [41–45]. This gelatinase appears similar to the type IV collagenase present in several tumor cell lines [41, 46], but is a distinct gene product from the 72 kDa gelatinase present in connective tissue cells [47–49] and cultured mesangial cells [50–52]. Since collagenase is relatively specific for collagen types I, II, III, which are not present in significant amounts in GBM, a major role for this metalloproteinase in GBM degradation appears unlikely. However, Lubec and coworkers [53, 54] have reported increased collagenase activity in kidneys obtained from rats with antiGBM antibody disease [53] and immune complex glomerulonephritis [54].

Gelatinases (type IV collagenases) are named because of their ability to degrade gelatin (denatured type I collagen) in vitro [42–45, 47, 48]. In vivo, gelatinases are postulated to play important roles in the degradation of type IV collagen, the major protein component of basement membranes [41, 45, 50–52, 55]. The ability of highly-purified human PMN gelatinase to degrade in vitro collagen types IV and V as well as fragments of type I (generated by collagenase) is well documented [41–46, 49]. PMN gelatinase can also degrade intact GBM at neutral pH (pH range 6.0 to 8.6) as shown by studies from our laboratory [56]. Compared to gelatinase (human PMN), collagenase (human fibroblasts) was nearly inactive in the degradation of intact GBM [56].

Several studies have extended these observations using stimulated intact neutrophils. Bray, Hume and Robinson [57] have reported that rabbit neutrophils degrade GBM in vitro at neutral pH. The amount of degradation was increased when the GBM was pretreated with antiGBM antibodies. Soya bean trypsin inhibitor and alpha-1-proteinase inhibitor (serine proteinase inhibitors) inhibited GBM degradation suggesting the involvement of elastase and/or cathepsin G. Vissers, Winterbourn and Hunt [58] have examined GBM degradation by neutrophils using "model systems" consisting of intact human neutrophils incubated with isolated human GBM impregnated with either nonspecific IgG or albumin-antialbumin immune complexes. GBM degradation was markedly inhibited by the serine proteinase inhibitor phenylmethane sulfonylfluoride (PMSF), suggesting that elastase and/or cathepsin G account for most of the GBM degradation in this system. We have shown [59] that GBM degradation by phorbol myristate acetate-stimulated PMN is mediated by activation of a metalloproteinase (possibly gelatinase) which is activated by hypochlorous acid (or a similar oxidant) generated by the PMN myeloperoxidase-H_2O_2-halide

system. Using their in vitro model of immune complex GBM injury (see above), Vissers and Winterbourn [60] estimated that neutrophil gelatinase accounted for approximately 30% of the GBM degradation with elastase/cathepsin G responsible for the remainder. In a subsequent study comparing GBM degradation by human neutrophils and monocytes, Vissers et al [61] observed that GBM degradation by monocytes was inhibited nearly equally by PMSF and o-phenanthroline, suggesting that both serine (elastase, cathepsin G) and metalloproteinases (gelatinase) were involved.

Taken together, these studies leave little doubt concerning the ability of leukocyte proteinases to degrade GBM in vitro. Coupled with the in vivo studies discussed below they provide strong support for proteinase-mediated GBM degradation as a potentially important mechanism of glomerular injury in those types of glomerular disease characterized by significant leukocyte infiltration.

GBM degradation in leukocyte-independent glomerulonephritis. Several types of glomerular disease, including both experimentally-induced and human glomerulonephritis, lack significant leukocyte involvement. Thus leukocytes can not serve as major sources of proteolytic enzymes for glomerular injury in these types of glomerular disease. Recent studies have documented the presence of GBM-degrading proteinases in freshly isolated glomeruli and cultured glomerular cells, raising the possibility that endogenous glomerular proteinases may be responsible for GBM damage in these types of glomerulonephritis. Although glomeruli contain proteinases from each major class, published studies to date suggest that endogenous glomerular metalloproteinases and cysteine proteinases may be the most important as mediators of GBM damage in leukocyte-independent GN.

Metalloproteinases. Lovett and coworkers have identified, characterized, and purified a gelatinase, which is present in the medium (but not the cells) obtained from cultured rat [50, 51] and human [52] mesangial cells. Similar to other matrix-degrading metalloproteinases, both the rat and human mesangial cell gelatinase are synthesized and secreted as inactive zymogens which can be activated in vitro by aminophenyl mercuric acetate (APMA) or limited proteolysis with trypsin [50–52]. The physiological activator(s) of latent gelatinase are unknown. The purified gelatinase from either species: had a molecular weight of 66 to 68 kDa; was optimally active at neutral pH; and degraded intact GBM, soluble type IV collagen, and gelatin but not fibronectin, albumin, proteoglycan, or interstitial collagen (types I, II, and III). These data suggest that the mesangial cell gelatinase is similar to the 72 kDa gelatinase found in connective tissue cells [47–49]. These workers also reported [52] that cultured human mesangial cells secrete a protein inhibitor of gelatinase with the characteristics of TIMP-1 (tissue inhibitor of metalloproteinases). TIMP-1 [62] and TIMP-2 [63–65] are functionally similar but distinct gene products thought to play major roles in the physiological control of gelatinase activity [11–13, 62]. Lovett and coworkers reported that immunohistochemical staining of glomeruli demonstrated the presence of both gelatinase and TIMP in the mesangial area of human biopsy specimens [52].

Studies from our laboratory [7, 8] have documented that glomeruli isolated from normal rats also contain one or more GBM-degrading metalloproteinases. Incubation of freshly isolated rat glomeruli with intact GBM at pH 7.5 resulted in significant GBM degradation as measured by hydroxyproline release [7]. GBM degradation was markedly inhibited by the metalloproteinase inhibitors, EDTA and o-phenanthroline, but unaffected by inhibitors of serine (PMSF, leupeptin, alpha-1-PI, SBTI) or cysteine proteinases (E-64, leupeptin), documenting that metalloproteinase activity was responsible for the GBM degradation. In a separate study [8] we have characterized a gelatin-degrading metalloproteinase activity present in freshly isolated rat glomeruli. Comparison of the properties of this metalloproteinase (membrane-associated; molecular weight = 116 to 120 kDa; lack of inhibition by TIMP, lack of activation by APMA) with those of other well characterized matrix metalloproteinases (such as, gelatinase, type IV collagenase, stromelysin) indicates that this glomerular metalloproteinase is distinct from these metalloproteinases including the mesangial cell gelatinase [50–52]. Whether the GBM and gelatin degrading proteinases present in isolated rat glomeruli are the same or separate enzymes is unclear at the present time.

Although direct evidence linking endogenous glomerular metalloproteinases to GBM damage (or other types of glomerular injury) in vivo has not been reported, their ability to act at neutral pH and to degrade GBM suggests an important role for these proteinases in glomerular pathophysiology.

Cysteine proteinases. Cysteine proteinases are a diverse class of lysosomal enzymes with a relatively broad substrate specificity and acid pH optima [66]. Among the best characterized of the cysteine proteinases are Cathepsins B, H, and L. The ability of highly purified cathepsins B and L to degrade intact GBM in vitro has been reported by our lab [67] as well as by Davies and coworkers [68–70]. In our studies [67], cathepsin L was more effective in GBM degradation than several other proteinases. In addition to intact GBM, purified cysteine proteinases have been shown in vitro to degrade purified GBM components including collagens types IV, and V (B and L: [69, 70], proteoglycan (B, H, and L: [69, 71]; and laminin, (B and L: [69].

Several lines of evidence, including enzyme activity [9, 72], immunohistochemistry [73, 74], and fluorescence microscopy [9], have established the presence of cathepsins B, H, and/or L in glomeruli isolated from normal rats. Furthermore, we have recently demonstrated the ability of isolated rat glomeruli to utilize endogenous cysteine proteinases to degrade intact GBM in vitro [9].

These studies document the ability of endogenous glomerular cysteine proteinases to degrade GBM and GBM components in vitro. However, most cysteine proteinases, including those documented to be present in glomeruli, are relativley inactive at neutral pH [9, 66, 67]. Thus special conditions (for example, an acidified microenvironment) must be envisioned for these proteinases to effectively act in the neutral pH of the glomerular capillary loop. Although such conditions have not been clarified, these data coupled with the beneficial effects of cysteine proteinase inhibitors in an experimental model of glomerular disease (see below) indicate an important role for cysteine proteinases in the pathogenesis of glomerular disease.

Urinary excretion of GBM components and proteolytic enzymes in glomerulonephritis

If proteinase-mediated GBM damage is indeed a mechanism of glomerular injury in vivo, one might expect to find increased amounts of GBM components and proteinase activity in the urine obtained from patients and animals with glomerulonephritis. Such results have been reported in both leukocyte-dependent and leukocyte-independent types of glomerulonephritis, including experimentally-induced antiGBM antibody disease [3, 75–77] and puromycin aminonucleoside-induced glomerulonephritis [78–81]. Similar results have also been reported for patients with various types of glomerulonephritis, including diffuse proliferative glomerulonephritis [82], systemic lupus and membranous glomerulonephritis [83], and acute post-strep glomerulonephritis [84]. Taken together, these studies provide further support for proteinase-mediated GBM damage as a potential mechanism of glomerular injury.

A summary of the evidence supporting GBM degradation as an important mechanism of proteinase-mediated glomerular injury is presented in Table 2.

Proteolysis of non-GBM proteins

In addition to the protein components of the GBM, a variety of structurally and/or functionally important intra- and extracellular proteins (for example, receptors, cytoskeletal proteins, enzymes, etc.) are susceptible to proteolysis by proteolytic enzymes. It is important to point out that proteolysis of such proteins may result in either activation or inactivation of the target protein. Of particular interest with respect to proteinase-mediated glomerular injury is the inactivation of proteinase inhibitors and the activation of latent proteolytic enzymes (zymogens). Although most of the examples presented below involve proteinases and target proteins from other tissues, the availability of similar proteinases and protein targets in glomeruli raises the possibility that these interactions may occur in glomeruli as well.

Activation of latent proteinases. The ability of proteolytic enzymes to activate other enzymes, often latent proteinases (zymogens) themselves, is well documented. Although all classes of proteolytic enzymes have been documented to activate one or more proteinases, activation by serine proteinases appears to be the most common and offers the greatest potential with respect to glomerular injury. Because of their many effects potentially relevant to glomerular injury, the activation of latent gelatinase (leukocyte or mesangial cell) and the conversion of plasminogen to plasmin are of particular interest in this regard.

Both elastase [85] and cathepsin G [42] have been reported to activate latent leukocyte gelatinase in vitro [see however, 86]. Although the mesangial cell gelatinase is a distinct gene product from the leukocyte gelatinase [46], the ability of a variety of agents (SDS, organomercurials, trypsin) to activate either of these gelatinases indicates similarities in their activation mechanisms and raises the possibility that elastase and/or cathepsin G may also activate latent mesangial cell gelatinase. Such interactions could represent an important mechanism for the activation of gelatinase in both leukocyte-dependent and independent types of glomerulonephritis.

Several lines of evidence suggest that plasmin, produced from plasminogen by plasminogen activators, may be an important mediator of glomerular injury. Intraglomerular fibrin deposition is thought to play an important role in the development and progression of several types of experimental as well as human glomerulonephritis [10]. Clearly the ability of the glomerulus to influence fibrin deposition via fibrinolysis may play an important role in this process. The presence of fibrinolytic activity in the glomerulus is well documented and apparently mediated by the conversion of plasminogen to plasmin [87–89]. Both tPA and uPA type plasminogen activators have been identified in glomerular endothelial, epithelial and/or mesangial cells [90–92]. In addition, Machovich and Owen [93] have reported that neutrophil elastase enhances the rate of plasminogen activation by two or more orders of magnitude by a combination of effects involving increased susceptability of plasminogen to plasminogen activators and inactivation of alpha-2-antiplasmin.

In addition to its role in fibrinolysis, plasmin has other effects of potential relevance to glomerular injury. The ability of plasmin to degrade components of the GBM, including proteoglycan [94]; the glycoproteins laminin and fibronectin [95–98]; and type IV collagen [99] has also been reported. Jones and coworkers [100, 101] have reported that glycoproteins markedly inhibit the degradation of collagen and elastin present in the extracellular matrix secreted by smooth muscle cells in culture and that plasmin, by degrading these glycoproteins, accelerated the rate of collagen and elastin degradation. Thus plasmin may accelerate the degradation of GBM collagen (by gelatinase or elastase for example) by removal of the glycoprotein components of GBM. In addition to these effects, plasmin may activate latent gelatinase. Based on the ability of plasmin to activate latent collagenase [11–13, 102–104] and apparent similarities in the activation mechanisms of latent gelatinase and latent collagenase by other agents [105], the possibility that plasmin may activiate latent gelatinase must also be considered. However, Nagase and coworkers [86] failed to observe activation of latent fibroblast gelatinase by purified plasmin.

The high concentration of plasminogen in the plasma, its continuous presence in the glomerulus, the production of plasminogen activators by glomerular cells, and the ability of plasmin to produce a variety of biological effects potentially related to glomerular injury suggest an important role for this proteinase as a mediator of glomerular injury.

Inactivation of proteinase inhibitors. Several inhibitors of proteolytic enzymes are well characterized proteins which are thought to play important roles in the regulation of proteinase activity in vivo. Such inhibitors include the plasma proteinase inhibitors such as alpha-1-proteinase inhibitor, alpha-2-antiplasmin, alpha-1-cysteine proteinase inhibitor, etc. [reviewed in 106], the cystatins, intracellular inhibitors of cysteine proteinases [reviewed in 107], and TIMP (tissue inhibitor of metalloproteinases) [63–65, reviewed in 62]. As proteins, these inhibitors are susceptible to degradation by proteolytic enzymes. A good example of the importance of proteinase inhibitors in vivo is demonstrated by the high incidence of emphysema, apparently mediated by neutrophil elastase, which occurs in patients with severe alpha-1-proteinase inhibitor (alpha-1-PI) deficiency [108]. Clearly, decreased levels of proteinase inhibitors (either systemic or localized to the pericellular microenvironment) potentially could result in glomerular injury via excess proteolytic enzyme activity. Proteolytic enzymes have been reported

to inactivate several proteinase inhibitors including C1 inactivator [109] and antithrombin III [110]. However, based on the effects of elastase, gelatinase, and plasmin on GBM components (as discussed above) and on the production of key metabolites (see below), inactivation of the inhibitors of these proteinases (alpha-1-PI, TIMP, and alpha-2-antiplasmin, respectively) is of particular interest here. Proteolytic inactivation of alpha-1-PI in vitro by cathepsin L [111], macrophage elastase [112], and a metalloproteinase secreted by PMA-stimulated human neutrophis [113] has been reported. Okada et al [114] have reported that in vitro human neutrophil elastase, trypsin, and chymotrypsin (but not cathepsin G, pancreatic elastase, or plasmin) effectively inactivate TIMP-1, a major physiological inhibitor of gelatinase and other matrix metalloproteinases [11–13, 62–65]. Neutrophil elastase has been reported to inactivate alpha-2-antiplasmin [109, 115]. That these or similar reactions take place in vivo is indicated by the observation that proteolyzed alpha-1-PI has been recovered from the sites of inflammation [116, 117]. Although the pathophysiological significance of these observations to glomerular injury remains to be established, the presence of several of these proteinases in glomeruli (either intrinsically or via infiltrating leukocytes) raises the possibility that inactivation of one or more proteinase inhibitors could result in increased activity of the corresponding proteinase with subsequent deleterious effects on glomerular structure and function. In keeping with this idea, it is interesting to note that several cases of glomerulonephritis associated with severe genetic deficiency of alpha-1-PI have been reported [118–121].

The ability of proteinases to activate latent proteinases and inactivate the corresponding proteinase inhibitors raises the possibility of a potentially damaging "synergism" as a result of these combined effects. For example, elastase has been reported to activate latent gelatinase [85] and to inactivate the major physiological gelatinase inhibitor, TIMP [41, 114]. Should these actions occur simultaneously in the same microenvironment, the net result would be a dramatic increase in the local activity of gelatinase and amplification of any effects (positive or negative) arising from the active enzyme. Clearly similar synergistic interactions can be postulated for other proteinase/proteinase inhibitor pairs such as plasmin and alpha-2-antiplasmin, and elastase and alpha-1-PI. Furthermore, it should be pointed out that such synergistic interactions can be effected by agents other than proteinases. For example, intact glomeruli, leukocytes, and cultured mesangial cells produce reactive oxygen metabolites (ROM) [reviewed in 122], agents which have been reported to activate latent gelatinase [123] and inactivate TIMP-1 [124].

A summary of the evidence supporting a role for proteinase-mediated degradation of non-GBM proteins as a mechanism of glomerular injury is presented in Table 2.

Proteinase-mediated alterations in glomerular metabolism and function

In vitro, proteolytic enzymes have been shown to alter the production by intact cells of a variety of key metabolites, autocrines, and immune mediators including: stimulation of the production of PAF (platelet activating factor) by elastase and cathepsin G [25]; stimulation of the release of adenine nucleotides by elastase [23]; stimulation of the synthesis of PGI_2 by thrombin, trypsin, and cathepsin G [22, 23]; stimulation of PGE_2 production by elastase [125]; stimulation of Ca^{2+} flux and IP_3 (inositol trisphosphate) synthesis by elastase [26] and thrombin [27]; and inhibition of thromboxane B_2 synthesis by thrombin [24]. Thrombin has been reported to also stimulate the production of uPA, tPA, and plasminogen activator inhibitor by cultured glomerular epithelial cells [29] and to increase the expression of endothelin in cultured mesangial cells [126]. However, of particular interest here is the ability of proteinases to modulate the production of cAMP and reactive oxygen metabolites (ROM), and to stimulate cell proliferation.

Proteolytic enzymes and the cAMP system. Several recent studies have suggested an important role for cyclic nucleotides in glomerular pathophysiology [127, 128]. In addition, extensive experimental evidence indicates that cAMP/cGMP may modulate inflammatory and immune responses in a variety of tissues [129, 130]. The ability of proteolytic enzymes, including trypsin, elastase, chymotrypsin, and acrosin to alter in vitro the activity of enzymes associated with the metabolism of cAMP and cGMP is well documented [131–137]. In addition, trypsin and thrombin have been reported to increase the cAMP content of intact fibroblasts [20] and lymphocytes [136]; and thrombin increases the cGMP content of cultured neuroblastoma cells [21]. These changes in cyclic nucleotide metabolism require active proteinases, are irreversible, and do not appear to result from inactivation of phosphodiesterases. Although the precise mechanism of action is presently unclear, cleavage of an inhibitory G protein has been postulated in at least one case [135]. Similar effects have been observed in freshly isolated glomeruli. Shah [138] has reported that thrombin and trypsin caused a marked increase in the cAMP content of isolated glomeruli. Thrombin had no effect on the cGMP content of glomeruli and no effect on either cAMP or cGMP content of tubules. Thrombin did not affect the cAMP phosphodiesterase activity of the glomeruli. The glomerular response to DIFP-treated thrombin was markedly reduced suggesting that proteolytic activity of thrombin is required for the response.

Role of proteolytic enzymes in the production of reactive oxygen metabolites. Several lines of evidence suggest that reactive oxygen metabolites (ROM) may play an important pathogenic role in leukocyte-dependent as well as leukocyte-independent types of glomerulonephritis [122]. Leukocytes, including PMN, monocytes, and macrophages are known to produce ROM in response to a variety of stimuli [139]. The ability of proteinase inhibitors to inhibit ROM production by stimulated leukocytes suggests an important role for proteolytic enzymes in this process. For example, the serine proteinase inhibitors PMSF, TLCK, TPCK, aprotinin, and SBTI all markedly inhibited ROM production by human PMN and monocytes stimulated with cytocalasin E or concanavalin A [140]. In addition, pretreatment of macrophages with proteolytic enzymes (elastase, trypsin, or and cathepsin G) resulted in enhanced ROM production following stimulation of the pretreated cells [141, 142].

Isolated glomeruli [143] as well as cultured mesangial cells [144] have also been shown to generate ROM. Although a role for proteinases in glomerular ROM production has not been reported, the similarities between ROM production by glomeruli and phagocytic cells suggest that proteinases may also play

a key role in glomerular ROM production. Indeed, Basci and Shah [28] have reported that treatment of freshly isolated glomeruli with trypsin or chymotrypsin results in the production of chemiluminescence, a phenomenon usually associated with ROM. In contrast to glomeruli, tubules had only a minor chemiluminescence response. Thrombin, bacterial collagenase and plasmin had no effect on ROM production by glomeruli.

Proteinase-mediated effects on cell proliferation. Several types of human and experimental glomerular diseases are characterized by abnormal proliferation of glomerular cells. Although the mechanisms responsible for such proliferation are unclear, recently published studies document the ability of thrombin to stimulate the proliferation of cultured glomerular cells. He et al [29] have reported that human alpha-thrombin promoted the proliferation of cultured human glomerular epithelial cells. This effect was inhibited by hirudin (which blocks both receptor binding and catalytic activity of thrombin) and DIPF (an irreversible thrombin inhibitor which does not block receptor binding) indicating that both receptor binding and catalytic activity are required to produce the effect. Abboud and coworkers [27] have reported that bovine thrombin caused a dose dependent increase in DNA synthesis (^{3}H-thymidine uptake) in cultured human mesangial cells. This effect was blocked by hirudin and also observed with a highly purified alpha-thrombin, suggesting that contaminating proteinases were not responsible for the stimulation of proliferation. The authors did not examine the effects of DIPF-treated thrombin. Thus the requirement for receptor binding, catalytic activity or both cannot be assessed.

Although only a limited number of studies have been carried out with isolated glomeruli or glomerular cells, these studies document the ability of proteolytic enzymes to modulate the production of cAMP and ROM (in both leukocytes and glomeruli) and to stimulate the proliferation of glomerular cells in culture and suggest that this may be an important mechanism of proteinase-mediated glomerular injury. A summary of the evidence supporting a role for proteinase-mediated alterations in glomerular metabolism is presented in Table 2.

In vivo studies supporting a pathogenic role for proteolytic enzymes in experimental models of glomerulonephritis

As discussed above, in vitro studies have documented several mechanisms by which proteolytic enzymes may potentially contribute to glomerular injury. Although such studies are essential to our understanding of the pathogenesis of glomerular injury, they do not *establish* a direct pathogenic role for proteolytic enzymes in glomerular disease. In contrast, recent in vivo studies involving leukocyte-dependent and leukocyte-independent models of glomerulonephritis provide direct evidence for a pathogenic of proteolytic enzymes in glomerular injury. However, because of the inherent complexity of in vivo systems, neither the specific proteinases nor their mechanism(s) of action have been established. Regardless of the mechanism(s), in vivo studies with experimentally-induced glomerulonephritis provide strong support for a pathogenic role for proteolytic enzymes in both neutrophil-dependent and neutrophil-independent glomerular disease.

Renal infusion of elastase or cathepsin G causes proteinuria in normal rats

One obvious corollary of the proteinase-mediated glomerular injury hypothesis is that exogenous administration of the appropriate proteolytic enzyme(s) should result in glomerular injury. Johnson et al [145] have reported that infusion of microgram quantities of either elastase or cathepsin G into the renal artery of rats resulted in marked proteinuria in the 24 hour period following the infusion. Only infusion of active elastase caused proteinuria although both active and irreversibly inactivated elastase localized equally in the glomerular capillary wall. Rats perfused with cationized IgG failed to develop proteinuria, establishing that neutralization of the glomerular anionic charge by the positively charged proteinases (pI 9 to 11) did not account for the proteinuria. Interestingly, glomeruli from kidneys perfused with active elastase were histologically normal with no foot process fusion and no evidence of inflammatory cell involvement or endothelial cell damage. Based on these data and the well-documented ability of elastase and cathepsin G to degrade GBM and GBM components in vitro (see above), the authors postulated that the proteinuria arises as a result of GBM damage by the infused proteinases. While a contribution from other effects of elastase and cathepsin G (as described above) can not be ruled out, this study documents the ability of exogenously administered proteinases to induce proteinuria in rats and provides strong support for proteinase-mediated GBM degradation as a mechanism of glomerular injury.

Beige mice do not develop antiGBM antibody-induced proteinuria

A second corollary of the proteinase-mediated glomerular injury hypothesis is that animals which lack a putative glomerular-damaging proteinase should not be susceptible to experimental models of glomerulonephritis involving that proteinase. Such evidence has recently been provided by Schrijver et al [146]. These workers compared glomerular injury and proteinuria resulting from experimentally-induced antiGBM antibody disease in control and Beige mice, the latter whose PMNs are deficient in elastase and cathepsin G. Injection of antiGBM antibodies (IgG fraction) into control mice resulted in a dose-dependent proteinuria during the 24 hour period immediately following the injection. In contrast, Beige mice injected with identical doses of antiGBM antibodies failed to develop proteinuria except at the highest dose of antiGBM IgG tested. With this dose, they excreted four times as much urinary protein as uninjected controls but still only 5% of that excreted in the antiGBM-treated normal mice. Immunofluorescence microscopy showed similar deposition of rabbit IgG and mouse complement component C3 in glomeruli from both groups of mice following treatment with antiGBM IgG. The failure of Beige mice to develop proteinuria was not due to lack of infiltrating PMN. EM demonstrated similar degrees of PMN attachment to the GBM as well as swelling and necrosis of endothelial cells in both groups. PMA-stimulated superoxide production was similar in PMN from Beige and control mice. Catalase (a scavenger of H_2O_2) and deferoxamine (an iron chelator which prevents the formation of hydroxyl radical) had no effect on the proteinuria in control mice treated with antiGBM IgG, indicating that reactive oxygen metabolites are

not involved in the development of proteinuria in this model. This study clearly demonstrates that Beige mice do not develop significant proteinuria during the heterologous phase of experimentally-induced antiGBM antibody disease despite a similar degree of PMN infiltration, and antibody and C3 deposition in their glomeruli. These latter observations are interesting since they demonstrate that deposition of antibody and complement as well as the presence of PMN is not, in itself, sufficient to cause proteinuria. These data, in combination with the in vitro and in vivo studies presented above, provide additional support for proteinase-mediated GBM damage as a mechanism of proteinuria in the neutrophil-dependent antiGBM antibody disease model of glomerulonephritis.

Effect of synthetic proteinase inhibitors on pathological changes in experimental glomerulonephritis

An additional corollary of the proteinase-mediated glomerular injury hypothesis is that inhibitors of putative glomerular-damaging proteolytic enzymes should block or attenuate the pathological changes associated with experimental models of glomerulonephritis involving those proteinases. Such studies have been reported for both leukocyte-dependent and leukocyte-independent models of glomerulonephritis.

Cysteine proteinase inhibitors reduce proteinuria in a neutrophil- and complement-independent model of antiGBM antibody disease. Studies from our laboratory have provided evidence that cysteine proteinases may play a pathogenic role in a complement- and neutrophil-independent model of antiglomerular basement membrane (GBM) antibody disease [72, 147]. Transepoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) is a specific, irreversible inhibitor of cysteine proteinases. It is: unreactive with serine, aspartic, or metalloproteinases; does not react at appreciable rates with low molecular weight thiols such as glutathione; does not react with nonproteolytic enzymes which contain a cysteine at their active site [148, 149]; and is relatively nontoxic [150]. In two separate studies, rats treated with E-64 and proteinuric doses of neutrophil- and complement-independent antiGBM IgG excreted significantly less urinary protein than rats receiving identical doses antiGBM IgG alone (E-64-treated: -43%, $P < 0.001$). This reduction in urinary protein excretion was accompanied by a marked decrease in the specific activity of cathepsins B and L in glomeruli and cortex isolated from rats treated with antiGBM IgG and E-64 compared to either saline-treated controls or rats treated with antiGBM IgG alone. Renal function, as assessed by BUN and plasma creatinine measurements, was unchanged in either experimental group compared to saline-treated controls.

In a subsequent study [151] we confirmed and extended these observations. Rats treated with trans-epoxysuccinyl-L-leucylamido-(3-methyl)butane (Ep475, an E-64 analog) and proteinuric doses of neutrophil- and complement-independent antiGBM IgG exhibited significantly reduced proteinuria (-45%, $P < 0.05$) compared to rats receiving identical doses of antiGBM IgG only. There was a significant reduction in the activity of both cathepsin B and cathepsin L in renal cortices obtained from Ep-475-treated rats compared to either saline-treated controls or rats treated with antiGBM IgG only. Administration of Z-Phe-Tyr(O-t-butyl)CHN_2 ($ZFYCHN_2$), a specific, irreversible inhibitor mainly of cathepsin L [152], caused a similar reduction in proteinuria (-56%, $P < 0.05$). This reduction in proteinuria was accompanied by a marked decrease (-84%) in the specific activity of renal cortical cathepsin L in $ZFYCHN_2$-treated rats. There was no significant change in: the activity of renal cortical cathepsin B; renal antiGBM antibody uptake; plasma urea nitrogen or plasma creatinine values in the $ZFYCHN_2$-treated rats compared to rats treated with antiGBM IgG only or saline-treated controls.

The mechanism(s) by which cysteine proteinases may contribute to glomerular injury is presently unclear. As discussed above, cysteine proteinases are present in normal glomeruli and can degrade GBM and GBM components in vitro under acid conditions. The extracellular location of the GBM suggests that the initial step(s) in GBM degradation must take place at the neutral pH presumed to exist at the GBM-plasma interface. Most cysteine proteinases, including cathepsins B and L, are relatively inactive in vitro at neutral pH, although we have observed significant degradation of GBM by endogenous glomerular cysteine proteinases at pH 6.5 [9]. However, it must be kept in mind that determination of pH optima in vitro are dependent on the substrate used as well as the specific conditions of the assay [153]. Furthermore, the ability of certain cells to acidify their pericellular environment [154] may provide a mechanism for lysosomal acid proteinases to function in an extracellular environment. Although calpain [155] and cathepsin S [156] are cysteine proteinases which are active at neutral pH, little is known concerning the presence of these enzymes in glomeruli or the potential role of these proteinases in GBM damage or other types of glomerular injury. Thus while a direct role for cysteine proteinases in GBM degradation remains a possibility, other mechanisms of cysteine proteinase-mediated glomerular injury must be considered including inactivation of proteinase inhibitors [111] and activation of latent proteinases as previously demonstrated for cathepsin B [102]. Regardless of the mechanism, the ability of specific cysteine proteinase inhibitors to reduce proteinuria in an experimental model of glomerulonephritis indicates an important role for one or more cysteine proteinases in this model of glomerular injury.

Serine proteinase inhibitors. Jennette et al [157] have reported a significant reduction (-69% to -89%) in glomerular necrosis in mice with horse apoferritin (HAF)-induced immune complex glomerulonephritis that had been treated with amidine-type inhibitors of trypsin-like proteinases (but which did not effectively inhibit the complement system). There was no difference between treated and non-treated groups in the amount or distribution of IgG, HAF, or C3, as measured by immunofluorescence staining. In long-term survival studies (33 days) only 3 of 9 inhibitor-treated mice died compared with 7 out of 10 mice in the group receiving HAF only.

Ikehara et al [158] have examined the effect of FUT-175 (6-amidino-2-naphthyl p-guanidinobenzoate dimethanesulphonate), a synthetic proteinase inhibitor of C1r and C1 esterase, thrombin, plasmin, kallikrein, and trypsin, on the development of lupus nephritis in $(NZBxNZW)F_1$ mice. High doses of FUT-175 (400 mg/kg/day) decreased the incidence of severe proteinuria (>300 mg/dl), improved renal function (as indicated by BUN), and decreased immune complex and C3 deposits and glomerular damage as assessed histologically. FUT-175 had no effect on ANA titer. Although these inhibitors are specific for serine proteinases, the fact that they inhibit several serine

Table 3. In vivo evidence for proteinase-mediated glomerular injury

Model	Animal	End point	Proteinase	Reference
Proteinase infusion	Normal rats	Proteinuria	Elastase and cathepsin G	145
AntiGBM antibody disease[a]	Beige mice	Proteinuria	Elastase and cathepsin G	146
AntiGBM antibody disease[b]	Normal rats	Proteinuria	Cathepsin L and cathepsin B	72, 147, 151
Immune complex GN	Normal mice	Glomerular necrosis	Serine proteinases	157
Lupus nephritis	(NZBxNZW)F_1 mice	Proteinuria, BUN, IC and C3 deposition	Serine proteinases	158

[a] Neutrophil-dependent model
[b] Neutrophil-independent model

proteinases precludes identification of the specific serine proteinase involved or the mechanism of injury.

A summary of the in vivo evidence supporting proteinase-mediated glomerular injury is presented in Table 3.

Conclusions and perspective

A large number of in vitro studies provide strong support for the concept that proteinase-mediated GBM damage may be an important mechanism of glomerular injury in leukocyte-dependent as well as leukocyte-independent types of glomerulonephritis. In addition, recent in vitro studies indicate that proteolytic enzymes may contribute to glomerular injury by mechanisms independent of GBM damage, including activation of latent enzymes (such as, plasminogen, metalloproteinases), inactivation of proteinase inhibitors (alpha-1-proteinase inhibitor, alpha-2-antiplasmin, and tissue inhibitor of metalloproteinases), and proteinase-mediated alterations in glomerular metabolism (cAMP, reactive oxygen metabolites, Ca^{2+} and IP_3) and cell proliferation. It is important to point out that most in vitro studies have been carried out in simplified systems which lack proteinase inhibitors and other potentially important modulators known to be present in vivo. Nonetheless, in vitro studies clearly document the potential of proteolytic enzymes to mediate glomerular injury in vivo and aid in the design and interpretation of in vivo experiments.

In vivo studies utilizing experimental models of glomerulonephritis also support an important role for proteolytic enzymes in both leukocyte-dependent and independent models of glomerular disease. The potential for synergistic interactions among proteinases, proteinase inhibitors, and other effectors (for example, reactive oxygen metabolites) complicates the interpretation of in vivo studies and precludes in most cases the definitive identification of the specific proteinase involved and the mechanism(s) by which it produces glomerular injury. Nonetheless, these studies are in good agreement with the in vitro studies and provide support for the proteinase-mediated glomerular injury hypothesis. Clearly, further in vivo studies are needed to establish which types of glomerular diseases involve proteolytic enzymes and the identity of the proteinase(s) involved. The increasing availability of a variety of specific proteinase inhibitors suitable for experimental use in vivo should greatly facilitate such studies.

Glomerular disease is a multifaceted phenomenon resulting from interactions among diverse biological processes. Our understanding of the role of proteolytic enzymes in these processes is just beginning. The recognition that proteolytic enzymes may mediate glomerular injury by mechanisms independent of GBM damage; that complex interactions can occur between proteolytic enzymes, their inhibitors, and other effector systems; and that glomerular-damaging proteolytic enzymes may originate from sources other than PMN represent important conceptual advances in our understanding of the potential role of proteinases in glomerular injury. These concepts should provide a useful framework for the design and interpretation of future studies and hopefully enhance our overall understanding of the pathogenesis of glomerular disease.

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Appendix. Abbreviations

PMN: polymorphonuclear leukocyte
GN: glomerulonephritis
ROM: reactive oxygen metabolites
APMA: aminophenylmercuric acetate
SBTI: soya bean trypsin inhibitor
alpha-1-PI: alpha-1-proteinase inhibitor
alpha-2-AP: alpha-2-antiplasmin
PMSF: phenylmethylsulfonylfluroide
TIMP: tissue inhibitor of metalloproteinases
tPA: tissue plasminogen activator
uPA: urokinase-like plasminogen activator
TLCK: tolsyllysyl chloromethyl ketone
TPCK: tolsylphenylalanine chloromethyl ketone
DIFP: diisopropyl fluorophosphate
E-64: trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane
Ep-475: trans-epoxysuccinyl-L-leucylamido-(3-methyl)butane
SDS: sodium dodecylsulfate
PMA: phorbol myristate acetate
PAF: platelet activating factor
ZFYCHN$_2$: Z-Phe-Tyr(O-t-butyl)CHN$_2$
IP$_3$: inositol triphosphate